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Laminin 411 and 511 promote the cholangiocyte differentiation of human induced pluripotent stem cells

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ABSTRACT

The drug discovery research for cholestatic liver diseases has been hampered by the lack of a well-established human cholangiocyte model. Functional cholangiocyte-like cells differentiated from human induced pluripotent stem (iPS) cells are expected to be a promising candidate for such research, but there remains no well-established method for differentiating cholangiocytes from human iPS cells. In this study, we searched for a suitable extracellular matrix to promote cholangiocyte differentiation from human iPS cells, and found that both laminin 411 and laminin 511 were suitable for this purpose. The gene expression levels of the cholangiocyte markers, *aquaporin 1* (*AQP1*), *SRY-box 9* (*SOX9*), *cystic fibrosis transmembrane conductance regulator* (*CFTR*), *G protein-coupled bile acid receptor 1* (*GPBAR1*), *Jagged 1* (*JAG1*), *secretin receptor* (*SCTR*), and *γ-glutamyl transferase* (*GGT1*) were increased by using laminin 411 or laminin 511 as a matrix. In addition, the percentage of AQP1-positive cells was increased from 61.8% to 92.5% by using laminin 411 or laminin 511. Furthermore, the diameter and number of cysts consisted of cholangiocyte-like cells were increased when using either matrix. We believe that the human iPS cell-derived cholangiocyte-like cells, which were generated by using our differentiation technology, would be useful for the drug discovery research of cholestatic liver diseases.

1. Introduction

Cholangiocytes, the epithelial cells lining intrahepatic bile ducts, are exposed to high concentrations of bile acids in the liver. Selective bile acid transporters expressed in cholangiocytes play important roles in the uptake of bile acids secreted from hepatocytes. Cholangiocytes control various properties of bile acids such as solubility, ionization, and micelle formation [1]. Cystic fibrosis (CF) is one of the diseases caused by cholangiocyte dysfunction. Patients of CF have the mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene, which is necessary for bile acid homeostasis. The CTFR mutations cause the cholangiocytes to lose their control of the above-mentioned bile acid properties [2]. Moreover, these cholangiocyte dysfunctions can lead to cholestasis and liver cirrhosis [2]. To develop effective drugs against cholestatic liver diseases caused by cholangiocyte dysfunction, such as CF, it is necessary to establish a cholangiocyte model which can be utilized for drug screening. However, it is hard

Abbreviations: AQP1, aquaporin 1; BMP, bone morphogenetic protein; BSA, bovine serum albumin; CFTR, cystic fibrosis transmembrane conductance regulator; EGF, epidermal growth factor; FBS, fetal bovine serum; FGF, fibroblast growth factor; GGT1, γ-glutamyl transferase; GPBAR1, G protein-coupled bile acid receptor 1; HBC, hepatoblast-like cells; HGF, hepatocyte growth factor; iPS, induced pluripotent stem; IL6, interleukin 6; JAG1, Jagged 1; LN, laminin; OsM, Oncostatin M; SCTR, secretin receptor; SOX9, SRY-box 9.

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to obtain primary human cholangiocytes. Furthermore, some functions of mouse and rat cholangiocytes are not equivalent to those of human cholangiocytes (e.g. mouse and rat cholangiocytes hardly express CFTR [3]). Therefore, the generation of a novel human cholangiocyte model is eagerly anticipated.

Because human induced pluripotent stem (iPS) cells have the potential to self-replicate and differentiate into almost all types of cells [4], the cells derived from human iPS cells have the potential to be used as novel tools such as for an *in vitro* model for drug discovery. Therefore, it is expected that cholangiocyte-like cells derived from human iPS cells would be a valuable tool. However, there is still room for improvement in the cholangiocyte differentiation efficiency of human iPS cells [5–7]. Thus, we considered that it is necessary to improve the differentiation method of cholangiocytes from human iPS cells. It is known that the optimization of not only culture media and growth factors but also extracellular matrix is important for selective cellular differentiation. Laminins are heterotrimeric extracellular matrix proteins which consist of an α-chain, a β-chain, and a γ-chain. There are five, four, and three genetic variants, respectively, and 15 chain combinations have been identified *in vivo*. One of the laminin isoforms has been shown to be a valuable tool for human iPS cell maintenance [8,9], hepatoblast self-replication [10] and hepatocyte-specific differentiation [11]. Previously, Tanimizu et al. have shown that the number of bile ductal structures and the bile duct lumen size were decreased in the livers of laminin α5-knockout mice [12], suggesting that laminin is an essential molecule in bile duct development. Therefore, we expected that cholangiocyte differentiation would be promoted by using particular isoform of laminin-rich extracellular matrix.

In this study, we attempted to generate a method for selective cholangiocyte differentiation. First, the hepatocyte-like cells differentiated from human iPS cells were cultured on various laminin isoforms to promote cholangiocyte differentiation. Second, we investigated the percentage of cholangiocyte marker-positive cells, and the diameter and number of cysts consisting of cholangiocyte-like cells.

2. Materials and methods

2.1. Human iPS cells

Human iPS cells generated from the human embryonic lung fibroblast cell line MCR5 were provided from the JCRB Cell Bank (Dotcom, JCRB Number: JCRB1327) [13,14]. Human iPS cells were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Millipore) with ReproStem (ReproCELL) supplemented with 10 ng/ml fibroblast growth factor (FGF) 2 (KATAYAMA Kogyo Kagaku).

2.2. Hepatoblast differentiation

Before the initiation of hepatocyte differentiation, human iPS cells were dissociated into clumps by using dispase (Roche Diagnostics) and plated onto BD Matrigel Basement Membrane Matrix Growth Factor Reduced (Becton, Dickinson and Company). These cells were cultured in the mouse embryo fibroblasts-conditioned medium for 3–4 days. The differentiation protocol for the induction of definitive endoderm cells, and hepatoblast-like cells was based on our previous reports with some modifications [15]. Briefly, in the definitive endoderm differentiation, human iPS cells were cultured with the L-WNT3A-expressing cell (CRL2647; ATCC)-conditioned RPMI1640 medium (Sigma) containing 100 ng/mL Activin A (R&D Systems), 1% GlutaMAX (Invitrogen), 0.2% fetal bovine serum (FBS), and 1 × B27 Supplement Minus Vitamin A (Invitrogen) for 4 days. For the induction of hepatoblast-like cells, the definitive endoderm cells were cultured with RPMI1640 medium containing 30 ng/mL bone morphogenetic protein 4 (BMP4) (R&D Systems) and 20 ng/mL FGF4 (R&D Systems), 1% GlutaMAX, and 1 × B27 Supplement Minus Vitamin A for 5 days.

2.3. Cholangiocyte differentiation

To induce cholangiocyte differentiation, the human iPS cell-derived hepatoblast-like cells were cultured in collagen gel for 14 days. To establish collagen gel plates, 500 μl collagen gel solution (consisting of 400 μl type I-A Collagen (Nitta Gelatin), 50 μl 10 × DMEM, and 50 μl of 200 mM HEPES buffer containing 2.2% $NaHCO_3$ and 0.05 M NaOH) was added to each well, and then the plates were incubated at 37 °C for 30 min. The human iPS cell-derived hepatoblast-like cells (5×10^4 cells) were resuspended in 500 μl cholangiocyte differentiation medium (the cholangiocyte differentiation medium: DMEM/F12 was supplemented with 2% FCS, 10 mM nicotinamide, 0.2 mM 2-phospho-L-ascorbic acid, 5.0 mM sodium pyruvate, 20 mM HEPES, 1 × GlutaMAX, 50 nM epidermal growth factor (EGF) (day 0–5 of cholangiocyte differentiation), 20 nM Interleukin-6 (IL-6) (day 5–10 of cholangiocyte differentiation), and 1 μM sodium taurocholate hydrate (day 10–14 of cholangiocyte differentiation)), and then mixed with 500 μl of the collagen gel solution and plated onto the basal layer of collagen. After 30 min, 1 ml of differentiation DMEM/F12 medium was added to the well. For the cholangiocyte differentiation experiments using laminin, 50 μg/ml of laminin was added to the cholangiocyte differentiation medium.

2.4. Real-time RT-PCR

Total RNA was isolated from human iPS cells and their derivatives using ISOGENE (NIPPON GENE). cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Invitrogen). Real-time RT-PCR was performed with SYBR Green PCR Master Mix (Applied Biosystems) using a StepOnePlus real-time PCR system (Applied Biosystems). Relative quantification was performed against a standard curve and the values were normalized against the input determined for the housekeeping gene, *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*). PCR primers sequences were obtained from qPrimerDepot (http://primerdepot.nci.nih.gov/).

2.5. Flow cytometer

Single-cell suspensions of the human iPS cell-derived cells were fixed with 4% PFA at 4 °C for 10 min, and then incubated with mouse anti-aquaporin 1 (AQP1) antibody (Abcam, ab9566), followed by Goat anti-Mouse IgG (H + L) Secondary Antibody, Alexa Fluor 488 conjugate (Thermo Fisher Scientific). Control cells were incubated with mouse anti-rabbit IgG antibodies (Santa Cruz Biotechnology), and then incubated with Goat anti-Mouse IgG (H + L) Secondary Antibody, Alexa Fluor 488 conjugate. Flow cytometry analysis was performed using a FACS LSR Fortessa flow cytometer (BD Biosciences).

2.6. Preparation of laminin E8 fragment

Recombinant E8 fragments were produced using a FreeStyle 293 Expression System (Invitrogen) as described previously [9,16–18].

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